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(54) Title: MODULATING TOLERANCE BY MODULATING FcγRIIB RECEPTOR SIGNALLING

(57) Abstract: The invention relates to the field of immunology and medicine. It particularly relates to a method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine. The invention provides a method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine comprising providing a ligand capable of modulating FcγRIIB receptor signalling.

# Modulating tolerance by modulating FcγRIIB receptor signalling

The invention relates to the field of immunology and medicine. It particularly relates to a method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine.

One of the characteristics of the immune system is the ability to distinguish between "self" and "non-self". Preferentially, the immune system of an individual should respond to all invaders ("non-self"), but not to molecules of the individual itself. An individual has multiple mechanisms to prevent auto-reactivity (the reaction of the immune system to "self" molecules) or in other words to ensure tolerance. One possible mechanism is clonal deletion, in which lymphocytes that recognize "self" molecules are eliminated. A second possibility is called clonal anergy, a process in which auto-reactive lymphocytes are inactivated. These auto-reactive lymphocytes are still present, are able to recognize an antigen, but antigen recognition does not lead to activation of the immune system. A third way to ensure tolerance to "self" molecules is the suppression by auto-reactive suppressor lymphocytes of auto-reactive lymphocytes, which recognize an antigen that leads to activation of the immune system. Disturbances of the immunological tolerance typically result in the activation of certain auto reactive lymphocytes, which in turn result in a reaction to "self"-components and the development of autoimmune disorders.

Two other properties of the immune system, specificity and memory, ensure a fast and strong reaction to a repeated contact with a "non-self" antigen. However, an immune response can also be developed to relative harmless components that invade the body (for example dust or food components). In the case of an improper regulation, repeated contact with harmless antigens leads to an excessive reaction of the immune system. As a result multiple effector mechanisms are activated which actually cause diseases or so-called allergic reactions, like for example hay fever.

Immunoglobin Fc receptors (FcR) constitute a family of surface molecules on haematopoietic cells capable of stimulating or inhibiting cellular responses upon cross linking by their ligand, antibody-antigen complexes (for review: see 1). Types I and III FcR for IgG (FcγRI and FcγRIII) are expressed primarily on cells of the myeloid lineage and can mediate effector functions such as phagocytosis, antibody-dependent cytotoxicity, and release of inflammatory mediators (2). Signaling through type I and III receptors leads to activating signals through intracellular ITAM (immunoreceptor tyrosine based activating) motifs and subsequent recruitment of Src and Syk tyrosine kinases and activation of PI3K and $PIP_3$-dependent kinases. This eventually leads to sustained activation by release of intracellular calcium and extracellular calcium influx (3). In contrast to this is the inhibitory signal provided by the type II receptor (Fc]γRII), which is the most widely distributed and abundant of the Fcγ receptors and is found on both myeloid and lymphoid cells like macrophages, granulocytes, B cells and even stimulated T cells. This receptor contains an ITIM (immunoreceptor tyrosine based inhibitory) motif, which, after coligation to an ITAM bearing receptor, recruits the inositol phosphatase SHIP. This leads to hydrolysis of $PIP_3$ and prevention of further activation of downstream kinases (4,5). Studies using mice mutated for the inhibitory FcγRII have shown that these receptors are involved in modifying the development and progression of autoimmune diseases by B cell responsiveness of myeloid cell activation. Absence of FcγRII renders mice susceptible to inducible forms of autoimmunity as seen with collagen-induced arthritis and Goodpasture's syndrome (6, 7, 8), but also to spontaneous autoimmunity (9, 10).

Through the induction of mucosal tolerance the body is prevented from harmful inflammatory reactions against nondangerous proteins such as food components. This naturally occurring immunological phenomenon has been described for the mucosae of the upper and lower airways (11, 12, 13) and the gastro-intestinal tract (14, 15) and involves clonal deletion and anergy of T

cells, but also active T cell mediated suppression (16, 17, 18, 19). The latter is especially found when tolerance is induced via the nasal mucosa.

The initial steps leading to the induction of tolerance, after an antigen is deposited on the mucosal surfaces, are still largely unknown. In case of nasal tolerance, it was recently demonstrated in mice that there is an absolute requirement for the organized lymphoid tissue of the nose draining lymph nodes (21). In a series of experiments in which the nose draining cervical lymph nodes were either removed or replaced by other lymph nodes through transplantation, it was not only demonstrated that the presence of cervical lymph nodes was necessary for tolerance induction, but also that this function could not be taken over by other lymph nodes when transplanted to this site (21).

Consequently, neither the uptake of antigen through mucosal surface nor the uptake and transport of antigen by the antigen presenting cells from the mucosal surface to the draining cervical lymph nodes seems to determine whether tolerance is induced or not.

Although involvement of lymph nodes in the induction of mucosal tolerance is evident, the biochemical machinery pathway, its components and its regulation in connection to the induction or prevention of tolerance are still unknown.

The present invention now identifies that FcγRIIB mediated signaling is crucial for the development of tolerance.

The present invention identifies genes that are unique to the cervical lymph node by performing a subtractive hybridization of cDNA from cervical lymph nodes (CLN) versus cDNA of peripheral lymph nodes (PLN). The gene that is predominantly overexpressed in CLN codes for IgG2b. This is confirmed by a higher percentage of $IgG2b^+$ $B220^+$ cells in CLN compared to any PLN. The invention further discloses a tolerance induction experiment using mice mutated in their FcγRIIb receptor which showed that the presence of IgG2b plays a role in tolerance induction via negative signaling through

FcγRIIb. In short, FcγRII -/- mice are incapable to adequately generate mucosally induced tolerance, which is for example mimicked by antibody mediated blocking of FcγRII signalling in normal mice. Furthermore, high levels of the FcγRII binding IgG2b isotype are found in the lymph nodes that are instrumental for tolerance induction and FcγRII signalling leads to altered antigen presentation by dendritic cells.

These experiments disclose that FcγRIIB mediated signaling is crucial for the development of (mucosally induced) tolerance. Modulation of this signalling modifies the tolerance response.

Thus, in a first embodiment the invention provides a method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine comprising providing a ligand capable of modulating FcγRIIB receptor signaling.

FcγRIIB receptor signaling is herein defined as the signaling brought on by binding of a ligand, for example an antibody-antigen complex, to the FcγRIIB receptor. The FcγRIIB receptor contains an ITIM (immunoreceptor tyrosine-based inhibitory) motif, which after coligation to an ITAM bearing receptor, recruits the inositol phosphatase SHIP. This leads to hydrolysis of $PIP_3$ and prevention of further activation of downstream kinases. Thus, by providing a ligand capable of modulating the FcγRIIB receptor signaling, the tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine is modulated.

A ligand of the FcγRIIB receptor is herein defined as a substance capable of modulating the FcγRIIB signaling and can either be an enhancer (agonist) or an inhibitor (antagonist) of FcγRIIB signaling. Binding of an enhancer to an FcγRIIB receptor induces a negative signaling and thereby further activation of downstream processes is prevented. In contrast, binding of an inhibitor to an FcγRIIB receptor inhibits negative signalling. An example of an enhancer of FcγRIIB signaling is IgG2b and an inhibitor of FcγRIIB

signaling is for example, as disclosed herein within the experimental part, the K9361 antibody.

In a preferred embodiment the invention provides a method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine comprising providing a ligand capable of modulating FcγRIIB receptor signaling, wherein said tolerance is modulated via mucosal administration of said ligand. Examples of routes of mucosal administration, are nasal or gastric administration.

In a more preferred embodiment the invention provides a method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine comprising providing a ligand capable of modulating FcγRIIB receptor signaling and further comprising said antigen. Preferably, said antigen is also provided by mucosal administration. By providing a ligand capable of modulating FcγRIIB receptor signaling together with an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine, the tolerance to said antigen is modulated.

In another embodiment the invention provides a method for inducing tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder comprising providing a ligand capable of inducing FcγRIIB receptor signalling and further comprising said antigen. As disclosed herein within the experimental part, IgG2b plays a role in tolerance induction via negative signalling through FcγRIIB. Mice, mutated in their FcγRIIB receptor reproducibly failed to show tolerance induction, when given tolerizing doses of ovalbumin (OVA) either intranasally or orally. Therefore, by providing a ligand capable of inducing FcγRIIB receptor signalling together with an antigen involved in an allergic reaction or in an autoimmune disorder, tolerance to said antigen is induced.

A method according to the invention is very useful for inducing tolerance to an antigen involved in an allergic reaction like asthma, eczema,

hay fever, urticaria or food allergy. Allergic reactions can be caused by a variety of allergens (or antigens, the terms are used interchangeably herein) for example from animal or plant origin, or by industrial compounds. Many allergens have been defined and are available in purified or recombinant forms. Common plant allergens include: wheat gliadin, birch allergen (bet v 1), timothy grass, peanut allergen or rubber latex (Hev b 7). Common allergens from animal origin include β-lacto globulin (cow-milk), ovalbumin (egg), cat allergen (Fel d) and allergens from house dust mite (Der p 1), Der f 1 and Der 2) and from storage mite (Lep d 2). Nickel allergy is caused by Ni-ions from instruments and jewelry ($NiCl_2$ is the experimental allergen). Until now, the effects of an allergic reaction can only be relieved by providing certain medications to a patient in need thereof, but a patient suffering from said effects cannot be cured permanently. By providing a patient suffering from the effects of an allergic reaction with a ligand capable of inducing FcγRIIB receptor signalling together with an antigen involved in said allergic reaction, the immune system is taught to ignore said antigen or in other words tolerance to said antigen is induced. A new contact with said antigen does, due to the induced tolerance, not provide an allergic reaction and thereby the effects of said allergic reaction are diminished or more preferably completely abolished. As long as the allergen that is causing a particular allergic reaction is known, tolerance to said allergen is induced by providing an individual, suffering from the effects of said allergic reaction, with said allergen in combination with a ligand capable of inducing FcγRIIB receptor signalling. It is clear that when an allergic reaction is caused by multiple allergens, more than one allergen is included in a method according to the invention.

A method according to the invention is also very useful for inducing tolerance to an antigen involved in an autoimmune disorder like rheumatoid arthritis, multiple sclerosis, Systemic lupus erythematosus, Sjögrens' syndrome, autoimmune diabetes, celiac disease or Crohn's disease. It is clear to a person skilled in the art that the list of autoimmune diseases to which

tolerance can be induced is endless as long as the causative antigen is provided in combination with a ligand capable of inducing FcγRIIB receptor signalling. Other examples of immune diseases, ranging from organ-specific to non organ-specific are Hashimoto's thyroiditis, primary myxoedema, thyrotoxicosis, pernicious anaemia, autoimmune atrophic gastritis, Addison's disease, , stiff-man syndrome, Goodpasture's syndrome, myasthenia gravis, pemphigus vulgaris, pemphigoid, sympathetic ophtalmia, phacogenic uvetis, autoimmune haemolytic anaemia, idiopathic thrombocytopenic purpura, idiopathic leucopenia, primary biliary cirrhosis, ulcerative colitis, dermatomyositis, scleroderma, mixed connective tissue disease or discoid lupus erythematosus. Examples of suitable antigens are SS-56 and Ro (Systemic lupus erythematosus, Sjögrens' syndrome), glutamic acid decarboxylase (diabetes), insulinoma antigen 2 (insuline), HSP60 and collagen (rheumatoid arthritis) or MOG and MBP (multiple sclerosis). By providing a patient in need thereof, with a ligand capable of inducing FcγRIIB receptor signalling together with an antigen involved in said autoimmune disease, the immune system is taught to ignore said antigen or in other words tolerance to said antigen is induced and thereby the effects of said autoimmune disease are diminished or more preferably completely abolished.

An antigen typically involves any substance capable of eliciting an immune response. An antigen in a method according to the invention can for example be provided as a proteinaceous substance, either being a full-length protein or a (immunogenic) peptide thereof. It is however important that such a peptide is correctly processed by the antigen-presenting machinery to ensure proper antigen presentation. Such peptides are therefore preferably provided as a polypeptide comprising at least 2 peptides preferably comprising flanking amino acid sequences representing antigen-processing sites. Examples of such a polypeptide and procedures how to arrive at such a polypeptide are known in the art. One example of a polypeptide is a string-bead polypeptide wherein peptides used as an antigen in a method according to the invention are

separated by amino acids. The amino acids separating the (different) peptides can be subjected to cleavage in a cell and thereby multiple (possibly, different) peptides are provided. Another example of a suitable type of antigens are glycolipids. Yet another example of a suitable type of antigen are haptens e.g. metal ions, like nickel.

In a preferred embodiment the invention provides a method for inducing tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder comprising providing a ligand capable of inducing FcγRIIB receptor signalling and further comprising said antigen, wherein said ligand comprises at least the Fc part of IgG2b and/or a functional derivative and/or a functional fragment thereof. As disclosed herein within the experimental part, the direct relationship between the IgG2b isotype and signalling through FcγRIIB was shown by testing the ability of interaction of IgG2b Fc portions with Fc receptors on dendritic cells and the effects on accessory molecule expression and IL-12 production. To this bone marrow derived dendritic cells were cultured on plates coated with IgG2b and in the presence or absence of the FcγRIIB specific antibody K9361. IgG2b cross-linking leads to enhanced expression of CD86 and IL-12 production, which is even further enhanced after blocking the inhibitory signalling through FcγRIIB. It is clear to a person skilled in the art that only the essential part or parts of such a ligand are required for the invention. Therefore, a functional derivative and/or functional fragment thereof is herein defined as a derivative and/or fragment capable of modulating FcγRIIB receptor signalling, possibly in different amounts.

In yet another embodiment the invention provides a method for preventing tolerance to an antigen used in a vaccine against an infection disease comprising providing a ligand capable of inhibiting FcγRIIB receptor signalling and further comprising said antigen. Preferably tolerance is prevented via mucosal administration of said ligand and said antigen. By providing a ligand capable of inhibiting FcγRIIB receptor signalling together

with an antigen used in a vaccine against an infection disease, the immune system is stimulated to respond to said antigen even under the circumstances of repeated contact with said antigen, or in other words tolerance to said antigen is diminished or more preferably completely abolished.

A method according to the invention is useful for preventing tolerance to an antigen used in a vaccine against an infection diseaselike influenza, Helicobacter pylori or pneumococcen. Other examples of infection diseases are cholera, rotavirus or HIV. It is clear to a person skilled in the art that the list of infection diseases to which tolerance can be prevented is endless as long as the causative antigen is provided in combination with a ligand capable of inhibiting FcγRIIB receptor signalling. In the art, multiple natural or recombinant antigens are available for such a method. A suitable ligand capable of inhibiting FcγRIIB receptor signalling, as disclosed herein within the experimental part, is an antibody capable of blocking said FcγRIIB receptor but which after binding to the FcγRIIB receptor does not lead to signalling. It is clear to a person skilled in the art that only the essential part or parts of such a ligand capable of inhibiting FcγRIIB receptor signalling, are required for the invention. Therefore, a functional derivative and/or functional fragment of a ligand is herein defined as a derivative and/or fragment capable of inhibiting FcγRIIB receptor signalling, possibly in different amounts.

Furthermore the invention provides a pharmaceutical composition comprising a ligand capable of inducing FcγRIIB receptor signalling and an antigen involved in an allergic reaction or in an autoimmune disorder. Preferably such a pharmaceutical composition comprises at least the Fc part of IgG2b and/or a functional derivative and/or a functional fragment thereof as said ligand.

Such a pharmaceutical composition is in particular provided for the induction of tolerance (preferably said tolerance is induced by mucosal administration) to an antigen involved in an allergic reaction or in an autoimmune disorder or for reducing the effects of an allergic reaction or the

effects of an autoimmune disorder. Such a pharmaceutical composition is more in particular provided for the induction of tolerance to an antigen involved in an allergic reaction like asthma, eczema, hay fever, urticaria or food allergy or in an autoimmune disorder like rheumatoid arthritis, multiple sclerosis, Systemic lupus erythematosus, Sjögrens' syndrome, autoimmune diabetes, celiac disease or Crohn's disease. Such a pharmaceutical composition can be administered to a patient in need thereof via different routes. One possible route of mucosal administration is nasally, because this provides an easy access to mucosal tissue. Furthermore, nasal application may therefore be favourable when therapeutic applications of mucosal tolerance are considered to prevent allergies and autoimmune disorders, since both IgE-production and delayed type hypersensitivity (DTH) can be efficiently down-regulated by nasal tolerance. Another route of administration is gastrally.

In yet another embodiment the invention provides a pharmaceutical composition comprising a ligand capable of inhibiting FcγRIIB receptor signalling and an antigen used in a vaccine against an infection disease. Preferably such a pharmaceutical composition comprises an antibody against the FcγRIIb receptor and/or a functional derivative and/or a functional fragment thereof. An example of such an antibody is K9361.

Said pharmaceutical composition is in particular provided for preventing tolerance to an antigen used in a vaccine against an infection disease (preferably said tolerance is induced via mucosal administration of said pharmaceutical composition). Examples of infection diseases are influenza, Helicobacter pylori or pneumococcen.

In yet another embodiment the invention provides a method for identifying a ligand of FcγRIIB receptor signalling by screening a compound library comprising

- culturing cells comprising a FcγRIIB receptor
- providing a testcompound to said cells
- testing medium surrounding said cells for cytokine and/or chemokine levels

Examples of useful cells are monocytic celllines like U937 which comprise a FcγRIIB receptor. It is clear to a person skilled in the art that cells or cell lines which do not comprise a FcγRIIB receptor can easily be provided with said FcγRIIB receptor by for example a transfection experiment. Preferably readout systems like bead-arrays are used to ensure a quick determination of different cytokine levels.

The invention will be explained in more detail in the following description, which is not limiting the invention.

## EXPERIMENTAL PART

**Mice**

BALB/c mice were obtained from Harlan (The Netherlands) and kept in our animal facility under routine laboratory conditions. For the transplantation experiments 6-8 weeks old female mice were used. FcγRIIb -/- mice on B6 background[10] and WT C57BL/6 were obtained from the University of Utrecht.

**RT-PCR for gamma-immunoglobulins, TGF-ß1 and IL-6**

Total RNA was purified from lymph nodes or sorted B-cells using Trizol (Gibco BRL). cDNA was synthesized from total RNA using oligo-dT primer (Gibco BRL) and MMLV reverse transcriptase (Gibco BRL). To perform semi-quantitative PCR for the different gamma-immunoglobulins the constant regions of immunoglobulins were aligned using the ClustalW algorithm (EMBL-EBI, UK). Immunoglobulin specific primers were designed across different constant region exons resulting in these primers:
IgG1 5' CCAGGGATTGTGGTTGTA, IgG1 3' GCAGGGAAAGCTGCACT,
IgG2b 5' TCCATGCAAGGAGTGTC, IgG2b 3' GCATGAGAAGGAATCTGT,
IgG3 5' CTAGAATACCCAAGCCCA, IgG3 3' GGACATTTGTTCACGAG.
For TGF-ß1 and IL-6 the following primers were used:
TGF-ß1 5' CGGGTGGCAGGCGAGAG, TGF-ß1 3'-
TCAGCAGCCGGTTACCAAGG,
IL-6 5' GTTCTCTGGGAAATCGTGGA, IL-6 3' TGTACTCAAGGTAGCTATGG.
Actin was used to quantify the amount of cDNA;
actin 5' TGGATTCCTGTGGCATCCATGAAAC,
actin 3' TAAAACGCAGCTCAGTAACAGTCCG.
For semi-quantitative PCR the templates were diluted from cDNA in 5-fold dilution steps. Cycling conditions on the thermocycler (MJ-research, Watertown, MT) were 94°C for 30 s, 50°C (IgG's) or 62°C (TGF-ß1) for 30 s,

72°C for 45 s, for 40 cycles, and for IL-6 94°C for 30 s, 56°C for 2 min, 72°C for 3 min, for 10 cycles followed by 94°C for 30 s, 56°C for 1 min, 72°C for 3 min, for 30 cycles.

**Cell staining and FACS analysis and antibodies**

Cultured dendritic cells or single cell suspensions of lymph nodes prepared by using a nylon mesh were washed in RPMI containing 2% FCSi and 0.01% Sodium azide (Merck) (FACS-buffer). Antibodies used were biotin-labelled rabbit-anti-mouse IgM, IgA, IgG1, IgG2a, IgG2b, IgG3, IgE (Zymed, San Fransisco, CA) and anti-CD86 (clone GL-1), anti-CD40 (clone 3/23), FITC conjugated anti-MHCII (clone M5/114), PE-conjugated anti-CD80 (clone 1G10). Subsequently, the cells were washed three times with FACS-buffer and when conjugate staining was necessary the cells were incubated on ice for 30 minutes with PE-conjugated donkey anti-rat Ig (Jackson Labs, Maine USA) or streptavidin-PE (Vector, Burlingame, CA). To gate for B-cells in lymph node suspensions samples were additionally stained with 6B2 (α-B220), coupled to Alexa488 (Molecular Probes, Eugene, OR). 7AAD (Molecular Probes) was used to stain for dead versus live cells.

After incubation the cells were washed, resuspended in FACS-buffer and fluorescence was measured using a FACScan™ (Beckton Dickinson, Mountain View, CA). Per sample 10000 events were analyzed. Cells that had been incubated with conjugate alone served as negative control.

For RT-PCR purified B-cells (> 90%) and T-cells (>95%) were obtained in a single step by sorting single suspensions made from lymph nodes, stained with 6B2-Alexa488 (α-B220) in the fluorescence 1 channel and GK1.5-PE (α-CD4, Pharmingen San Diego, CA) and 53-6.7-PE (α-CD-8, Pharmingen) in the fluorescence 2 channel on a FACStar Plus (BectonDickinson, Mountain View, CA).

The K9361 hybridoma specific for the Ly17.1 allele of the FcγRIIb[23] was obtained from Dr. Marc Daeron and used as affinity purified Ig. The preparation of this hybridoma is described in an article from Kimura et al.[51].

**Transplantation of lymph nodes**

Transplantation of cervical or peripheral LN was performed as previously described[21]. In brief, brachial or cervical lymph nodes were removed aseptically and kept in sterile RPMI on ice. In the acceptor animals all cervical lymph nodes were removed and a superficial cervical or a brachial lymph node was placed at the site of the internal jugular lymph node. It has previously been shown that already after 1 week afferent lymphatics and blood supply are restored[24] and after 3 weeks tolerance could be induced in the presence of transplanted cervical lymph nodes[21]. After 3 and 30 weeks transplanted lymph nodes were removed and analyzed for isotype-specific B-cells.

**Tolerance induction and DTH**

For induction of nasal tolerance FcγRIIb -/- mice and WT C57BL/6 mice received three doses of 100 μg OVA / 10 μl saline intranasally on three consecutive days[19,21]. For oral tolerance induction mice received a single feed of 25 mg OVA (OVA type VII, Sigma Chemical Co, St. Louis, MO) intragastrically. Eight days after the last intranasal or oral administration the mice were sensitized by injecting 100 μg OVA / 25 μl saline / 25 μl incomplete Freund's adjuvans (IFA) subcutaneously in the tail base. As challenge for the DTH-response 10 μg OVA / 20 μl saline was injected intradermally in the auricle of the ear five days later. The increase of ear thickness was measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan) at 24 hours after the challenge and compared to the ear thickness as measured before OVA injection.

**Culturing of Dendritic cells**

Bone marrow derived dendritic cells (BM-DC) were cultured as described Lutz et al.[25]. In short, on day 0 femurs and tibia of C57Bl/6 mice were flushed, the resulting bone marrow suspension was retrieved and passed over a 70 μm cell-strainer (Becton Dickinson, Franklin Lakes, NJ) to obtain a single cell suspension. The cells were seeded at $2x10^6$ per dish in IMDM supplemented 20ng/ml recombinant murine GM-CSF (supernatant of X63 myeloma cells tranfected with murine GM-CSF cDNA) and 20ng/ml recombinant murine IL-4 (kind gift of Schering-Plough, Dardilly, France) (rmIL-4). On day 3, 10 ml of fresh IMDM containing 20ng/ml rmGM-CSF and 20ng/ml rmIL-4 was added. On day 6, half of the culture supernatant was collected and centrifuged, the pellet resuspended in 10ml fresh IMDM containing 10ng/ml rmGM-CSF and 10ng/ml rmIL-4, and added to the original plates. On day 8, the non-adherent cells consisting of immature and mature dendritic cells were harvested and used for subsequent experiments.

D1 cells, a kind gift of Dr. P. Ricciardi-Castagnoli, were cultured as described previously[26]. In short, D1 cells were seeded at 1.5- 2.0x $10^6$ cells per dish in IMDM supplemented with R1 medium to obtain a final concentration of 20ng/ml GMCSF. The cells were harvested after 3 or 4 days of culture and used for propagation or subsequent experiments.

**In vitro IgG2b blocking**

Dendritic cells were washed and incubated in suspension with K9361 mAb or ED6 mAb at a concentration of 10μg/ml in PBS at room temperature during 60 min. After incubation the cells were centrifuged and resuspended in IMDM. Twenty-four well plates (Greiner) were coated with purified mouse IgG2b anti-TNP (Pharmingen, San Diego, CA) 10μg/ml in PBS or PBS as a control at 37°C. After 60 min incubation the coating solution was removed and the dendritic cells were seeded in the wells at $1x10^6$/ml in the presence or absence of 10μg/ml K9361 or ED6. After 60 min incubation the cells were

stimulated with LPS from *E. coli* 0111:B4 (Difco, Detroit, MI) at a concentration of 1 or 10 ng/ml. The cells were cultured at 37°C and 5% $CO_2$ and after 24h the supernatant was collected, stored at -80°C, and the cells were recovered for further analysis.

**IL-12 p40 determination**

The concentration of IL-12p40 in the culture supernatants was determined by ELISA (Biosource, Nivelles, Belgium), which was performed according to the instructions of the manufacturer.

# RESULTS

FcγRIIb, which is for example expressed on dendritic cells of cervical as well as peripheral lymph nodes (results not shown), is the only Fc-receptor with a negative immunoregulatory ability due to the presence of an immunoreceptor tyrosine-based inhibitory (ITIM) motif in its cytoplasmic domain[2]. Phosphorylated ITIM serve as docking sites for the SH2-containing phosphatases which then inhibit signal transduction.

To determine the involvement, if any, of the FcγRIIB receptor in the induction of tolerance, induction experiments were performed using mice mutated in their FcγRIIb receptor. As is shown in Fig. 1, FcγRIIb -/- mice reproducibly failed to show tolerance induction, when given tolerizing doses of OVA either intranasally or orally.

**Increased expression of IgG2b in mucosal lymph nodes**

Semiquantitative PCR was used to look at the expression of IgGs in various lymph nodes in which we initially focussed on cervical, nose draining lymph nodes versus peripheral nodes. Since the genes encoding the different gamma-immunoglobulins are highly homologous, semiquantitative PCR was preferred over Northern blotting for the detection of IgG expression levels. Semiquantitative PCR on total lymph node material showed that IgG2b was expressed at approximately 125-fold higher levels in the cervical lymph nodes relative to the peripheral lymph nodes (Fig. 2A). Compared to expression of IgG2b, expression of IgG1 and IgG3 mRNA was moderately increased in the cervical lymph nodes (Fig. 2B).

The increase in IgG2b expression in the cervical lymph node could be due to i) higher percentages of total B-cells, ii) a specific increase in the number of IgG2b positive B-cells, or iii) increased synthesis of IgG2b per cell. To discriminate between these possibilities the numbers of B cells in cervical and peripheral lymph nodes were determined and the contribution of the

various isotypes was analyzed. As shown in Figure 3 the distribution of the total number of B cells in cervical and peripheral lymph nodes was not different, as determined by B220 expression. However, in cervical lymph nodes there was a significantly higher proportion of B cells expressing the IgG2b isotype. This was confirmed by isolation of $B220^+$ B cells from the various lymph nodes using FACS sorting (Fig. 4). Using cDNA from these cells in a semiquantitative PCR IgG2b transcripts could not be detected in B-cells from peripheral lymph nodes, but B-cells from the cervical lymph node as well as the mesenteric lymph node expressed IgG2b. Transcripts for IgG1 and IgG3 were also found, but not preferentially expressed in the mucosa draining lymph nodes (Fig. 4). The abundant IgG2b expression in cervical compared to peripheral lymph nodes is therefore correlated to a higher number of IgG2b positive B-cells.

**Upstream effects that lead towards IgG2b synthesis**

What could be the underlying cause for such abundant expression of IgG2b? Several switch factors, like TGF-ß1 and IL-6, are associated with isotype switching and differentiation of B cells to the synthesis of IgG2b antibodies. Especially TGF-ß1 is considered as a main switch factor for IgG2b[27]. TGF-ß1 can be produced by many cell types, including T cells. We therefore first analyzed whole lymph node preparations for the production of TGF-ß1 by semi-quantitative PCR. However, no differences were found in whole tissue cDNA from cervical versus peripheral lymph nodes (results not shown). When T-lymphocytes from the two types of lymph nodes were sorted and analyzed for their level of TGF-ß1 by semiquantitative PCR no differences in expression could be found between T-cells from cervical and peripheral lymph nodes (results not shown).

Transcripts for IL-6, a cytokine also associated with differentiation of B cells and the isotype switching into IgG2b[28,29] were readily found in all

lymph nodes but no differences were found between the different types of lymphoid tissue (results not shown).

**The role of the drainage area on the expression of IgG2b**

The enhanced local IgG2b expression together with the unique capacity to confer immunolocal tolerance may be intrinsic to the mucosa draining lymph node or may be depending on a combination of lymph node organization and draining area. To reveal to what extent the draining area is responsible for the observed predominance of IgG2b expression, transplantation experiments were performed. Cervical and peripheral (brachial) lymph nodes were transplanted into the site of a cervical node and analyzed after three and 30 weeks for the presence of IgG2b positive B220 cells. After three as well as 30 weeks the percentage of IgG2b positive B-cells was similar for the transplanted cervical and transplanted brachial lymph node (Fig. 5). From these results it was concluded that the increased level of IgG2b expression at the site of the cervical lymph node was predominantly caused by effects of the drainage site, possibly related to the influx of specific factors or cells from the mucosa and submucosal tissues.

**Effect of FcγRIIB mediated signaling on the phenotype of dendritic cells**

To determine a relationship between the IgG2b isotype, signalling through FcγRIIB and decreased dendritic cells (DC) functions, cells of the D-1 dendritic cell line were stimulated *in vitro* with crosslinked IgG2b in the presence of low doses of LPS and the effects on accessory molecules and IL-12 secretion were investigated.

After 24h of stimulation with cross-linked IgG2b in the presence of low concentrations of LPS the expression of CD80, CD86, CD40 and MHC class II was increased on D-1 cells and the IL-12p40 release was enhanced when compared to D-1 cells incubated with LPS alone (Fig. 6 and 7). When the

signaling via the FcγRIIB on D-1 was neutralized using the mAb K9361, stimulation with cross-linked IgG2b and LPS increased the expression of CD86 and CD40 (Fig 6) and further enhanced the IL-12p40 secretion (Fig 7), whereas the expression of CD80, MHCII and CD11c were not affected (not shown). Stimulation of D-1 cells with cross-linked IgG2b and LPS in the presence of an irrelevant isotype control mAb did not increase the expression of CD86 and CD40 and slightly reduced the IL-12p40 release by the cells (Fig 7.)

## DESCRIPTION OF FIGURES

**Figure 1 Mucosal tolerance induction fails in FcγRIIb -/- mice.**

For induction of nasal tolerance or oral tolerance FcγRIIb -/- mice (black bars) and WT C57BL/6 mice (open bars) received three doses of 100 µg OVA / 10 µl PBS intranasally on three consecutive days[19,21], or a single intragastric feed of OVA, respectively. Eight days after the last intranasal or oral administration the mice were sensitized by injecting 100 µg OVA / 25 µl PBS / 25 µl incomplete Freund's adjuvans (IFA) subcutaneously in the tail base. As challenge for the DTH-response 10 µg OVA / 20 µl PBS was injected intradermally in the auricle of the ear five days later. Results are from a representative experiment and are expressed as the mean increase (+SD) of ear thickness of 7 animals per group at 24 hours after the challenge compared to the ear thickness as measured before OVA injection. * indicates $p < 0.01$.

**Figure 2 Increased expression of IgG2b mRNA in cervical lymph nodes.**

Cervical and peripheral lymph nodes were isolated and expression of mRNA was analyzed by semiquantitative PCR. Actine was used for normalization of mRNA levels. A) expression of IgG2b, B) expression of IgG1 and IgG3.

**Figure 3 Cervical lymph nodes contain relatively more IgG2b$^+$ B220$^+$ cells than peripheral lymph nodes.**

Lymphocytes were isolated from lymph nodes (LN) of untreated mice and stained for B-cells (B220) and the immunoglobulin isotypes as indicated. 7AAD was used to stain dead cells. The isotype specific cells were gated for B220 and living cells and are expressed as percentages.

**Figure 4 B220$^+$ cells from mucosa draining lymph nodes produce more IgG2b mRNA than B220$^+$ cells from peripheral lymph nodes.**

$B220^+$ cells were sorted from cervical and peripheral lymph nodes, and analyzed with semiquantitative RT-PCR for the expression of IgG2b, IgG1, IgG3. Actin was used for normalization of mRNA levels.

**Figure 5 $IgG2b^+$ $B220^+$ cells after lymph node transplantation**

After transplantation to the cervical region the cervical and the brachial lymph nodes contain an equal percentage of $IgG2b^+$ $B220^+$ cells. Three weeks after transplantation, transplanted cervical (Ce) and brachial (Br) lymph nodes were isolated from untreated mice. Non-transplanted cervical lymph nodes (CLN) from control mice served as control. As an internal standard for all mice the non-transplanted brachial lymph nodes (BLN) were extracted. Lymphocytes were stained for B-cells (B220) and immunoglobulin isotype. 7AAD was used to stain dead cells. The isotype specific cells were gated for B220 and living cells. A) percentage of $B220^+$ cells, B, percentage of IgM specific B-cells, C) percentage of IgG2b specific B-cells.

**Figure 6 Effect of FcγRIIB mediated signaling on the expression of accessory molecules on D-1 cells.**

Plates were coated with purified mouse IgG2b anti-TNP or PBS as a control. DC that had been incubated in suspension of 10μg/ml anti-FCγRIIB or control mAb were seeded into the wells at $1x10^6$ cells/ml in the presence or absence of an additional 10μg/ml anti-FCγRIIB or control mAb. After 60 min of incubation the cells were stimulated with 1 or 10 ng/ml LPS. After 24h of culture the cells were analyzed by flow cytometry. Results are representative of three separate experiments.

**Figure 7 Effect of FcγRIIB mediated signaling on the IL-12 release by DC.**

Plates were coated with purified mouse IgG2b anti-TNP or PBS as a control. DC that had been incubated in suspension of 10μg/ml anti-FCγRIIB or control

mAb were seeded into the wells at $1x10^6$ cells/ml in the presence or absence of an additional 10μg/ml anti-FCγRIIB or control mAb. After 60 min of incubation the cells were stimulated with 1 or 10 ng/ml LPS. After 24h of culture the concentration of IL-12p40 was determined in the supernatant. Results are representative of three separate experiments. Open bars represent stimulation with 1ng/ml LPS; black bars are stimulated with 10ng/ml LPS.

## REFERENCES

1. Ravetch JV, Bolland S. IgG Fc receptors *Annu Rev Immunol.* 19, 275-90 (2001)
2. Daeron, M. Structural bases of Fc gamma R functions. *Int Rev Immunol.* **16,** 1-27 (1997)
3. Weiss A, & Littman DR Signal transduction by lymphocyte antigen receptors. *Cell* **76**, 263-274 (1994)
4. Ono M, Bolland S, Tempst P, & Ravetch JV, Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc-gamma RIIB. *Nature* **383,** 263-266 (1996)
5. Bolland, S., & J. V. Ravetch.. Inhibitory pathways triggered by ITIM-containing receptors. *Adv Immunol.* **72**, 149-77 (1999)
6. Yuasa, T., S. Kubo, T. Yoshino, A. Ujike, K. Matsumura, M. Ono, J. V. Ravetch, & T. Takai. Deletion of fcgamma receptor IIB renders H-2(b) mice susceptible to collagen-induced arthritis. *J Exp Med.* **189,** 187-94 (1999)
7. Nakamura A, Yuasa T, Ujike A, Ono M, Nukiwa T, Ravetch J, & Takai T, Fcγ receptor IIB deficient mice develop God[pasture's syndrome upon immunization with type IV collagen: a novel murine model for autoimmune glomerular basement menmbrane disease. *J. Exp. Med.* **191**, 899-905 (2000)
8. Kleinau S, Martinsson P, & Heyman B. Induction and suppression of collagen-induced arthritis is dependent on distinct Fcγ receptors. *J. Exp. Med.* **191**, 1611-1616 (2000)
9. Pritchard NR, Cutler AJ, Uribe S, Chadban SJ, Morley BJ & Smith GC, Autoimmune-prone mice share a promoter haplotype associated with redeuced expression and function of the Fc receptor FcγRII. *Current Biol.* **10**, 227-230 (2000)

10. Takai T, Ono M, Hikida M, Ohmori H & Ravetch JV, Augmented humoral and anaphylactic responses in FcγRII- deficient mice *Nature* **379** 346-349 (1996)

11. Barchan, D., M. C. Souroujon, S. H. Im, C. Antozzi, & S. Fuchs.. Antigen-specific modulation of experimental myasthenia gravis: nasal tolerization with recombinant fragments of the human acetylcholine receptor alpha-subunit. *Proc Natl Acad Sci USA*. **96**, 8086-91 (1999)

12. Hoyne, G. F., R. E. O'Hehir, D. C. Wraith, W. R. Thomas, & J. R. Lamb. Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med.* **178**, 1783-8 (1993)

13. Joosten, LA, Coenen-de Roo CJ, M. M. Helsen, E. Lubberts, A. M. Boots, W. B. van den Berg, and A. M. Miltenburg. Induction of tolerance with intranasal administration of human cartilage gp-39 in DBA/1 mice: amelioration of clinical, histologic, and radiologic signs of type II collagen-induced arthritis. *Arthritis Rheum*. **43**, 645-55 (2000)

14. Faria AM, & Weiner HL, Oral tolerance: mechanisms and therapeutic applications. *Adv Immunol*. **73**, 153-264 (1999)

15. Strobel S, & Mowat AM, Immune responses to dietary antigens: oral tolerance. *Immunol Today* **19**, 173-81 (1998)

16. Harrison L, Dempsey-Collier CM, Kramer DR & Takahashi K, Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. *J Exp Med.* **184**, 2167-74 (1996)

17. Ke Y, & Kapp JA, Oral antigen inhibits priming of CD8+ CTL, CD4+ T cells, and antibody responses while activating CD8+ suppressor T cells. *J Immunol*. **156**, 916-21 (1996)

18. Tsitoura, D. C., R. H. DeKruyff, J. R. Lamb, and D. T. Umetsu. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4+ T cells. *J Immunol.* **163**, 2592-600 (1999)

19. van Halteren, A. G., M. J. van der Cammen, D. Cooper, H. F. Savelkoul, G. Kraal, & P. G. Holt.. Regulation of antigen-specific IgE, IgG1, and mast cell responses to ingested allergen by mucosal tolerance induction. *J Immunol.* **159,** 3009-15 (1997)

20. Tian, J., M. A. Atkinson, M. Clare-Salzler, A. Herschenfeld, T. Forsthuber, P. V. Lehmann, and D. L. Kaufman. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J Exp Med.* **183,** 1561-7 (1996)

21. Wolvers, D. A., C. J. Coenen-de Roo, R. E. Mebius, M. J. van der Cammen, F. Tirion, A. M. Miltenburg, and G. Kraal.. Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39. *J Immunol.* **162**, 1994-8 (1999)

22. Wolvers, D. A., M. J. van der Cammen, and G. Kraal.. Mucosal tolerance is associated with, but independent of, up-regulation Th2 responses. *Immunology* **92,** 328-33 (1997)

23. Holmes L, Palfree RGE, Hämmerling U, & Morse III HC, Alleles of the Ly-17 alloantigen define polymorphisms of the murine IgG Fc receptor. *Proc. Natl.Acad. Sci. USA* **82**, 7706-7710 (1985)

24. Mebius, R. E., J. Breve, G. Kraal, and P. R. Streeter. Developmental regulation of vascular addressin expression: a possible role for site-associated environments. Int Immunol. 5:443-9 (1993)

25. Lutz MB, Kukutsch N, Ogilvie ALJ, Rößner S, Koch F, Romani N, & Schuler G, An advanced culture method for generating large quantities of highly pure dendritic cells from mouse. *J. Immunol. Methods* **223**, 77-92 (1999)

26. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, Zimmermann VS,

Davoust J, Ricciardi-Castagnoli P, Maturation stages of mouse dendritic

cells in growth factor-dependent long-term cultures. *J Exp Med* **185**, 317-28 (1997)

27. McIntyre, T. M., D. R. Klinman, P. Rothman, M. Lugo, J. R. Dasch, J. J. Mond, and C. M. Snapper. Transforming growth factor beta 1 selectivity stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. *J Exp Med.* **177**:1031-7 (1993)
28. Kopf, M., S. Herren, M. V. Wiles, M. B. Pepys, and M. H. Kosco-Vilbois. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. *J Exp Med.* **188**:1895-906 (1998)
29. Splawski, J. B., L. M. McAnally, and P. E. Lipsky. IL-2 dependence of the promotion of human B cell differentiation by IL-6 (BSF-2). *J Immunol.* **144**:562-9 (1990)
30. Nakamura, A., T. Yuasa, A. Ujike, M. Ono, T. Nukiwa, J. V. Ravetch, and T. Takai. Fcgamma receptor IIB-deficient mice develop Goodpasture's syndrome upon immunization with type IV collagen: a novel murine model for autoimmune glomerular basement membrane disease. *J Exp Med.* **191**:899-906 (2000)
31. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity.* **11**:141-51 (1999)
32. Ding, A., C. F. Nathan, J. Graycar, R. Derynck, D. J. Stuehr, and S. Srimal. Macrophage deactivating factor and transforming growth factors-beta 1 -beta 2 and -beta 3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. *J Immunol.* **145**:940-4 (1990)
33. Pelton, R. W., M. D. Johnson, E. A. Perkett, L. I. Gold, and H. L. Moses. Expression of transforming growth factor-beta 1, -beta 2, and -beta 3 mRNA and protein in the murine lung. *Am J Respir Cell Mol Biol.* **5**:522-30 (1991)

34. Ahuja, S. S., F. Paliogianni, H. Yamada, J. E. Balow, & D. T. Boumpas.. Effect of transforming growth factor-beta on early and late activation events in human T cells. *J Immunol.* **150**:3109-18 (1993)

35. Dubois, C. M., F. W. Ruscetti, E. W. Palaszynski, L. A. Falk, J. J. Oppenheim, and J. R. Keller. Transforming growth factor beta is a potent inhibitor of interleukin 1 (IL-1) receptor expression: proposed mechanism of inhibition of IL-1 action. *J Exp Med.* **172**:737-44 (1990)

36. Kehrl, J. H., L. M. Wakefield, A. B. Roberts, S. Jakowlew, M. Alvarez-Mon, R. Derynck, M. B. Sporn, and A. S. Fauci.. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med.* **163**:1037-50 (1986)

37. Ludviksson, B. R., D. Seegers, A. S. Resnick, and W. Strober.. The effect of TGF-beta1 on immune responses of naive versus memory CD4+ Th1/Th2 T cells. *Eur J Immunol.* **30**:2101-11 (2000)

38. Perlmutter, R. M., D. Hansburg, D. E. Briles, R. A. Nicolotti, and J. M. Davie. Subclass restriction of murine anti-carbohydrate antibodies. *J Immunol.* **121**:566-72 (1978)

39. Bouchard, C., W. H. Fridman, and C. Sautes. Mechanism of inhibition of lipopolysaccharide-stimulated mouse B-cell responses by transforming growth factor-beta 1. *Immunol Lett.* **40**:105-10 (1994)

40. Garcia, B., R. Rodriguez, I. Angulo, A. W. Heath, M. C. Howard, and J. L. Subiza. Differential effects of transforming growth factor-beta 1 on IgA vs. IgG2b production by lipopolysaccharide-stimulated lymph node B cells: a comparative study with spleen B cells. *Eur J Immunol.* **26**:2364-70 (1996)

41. Lucas, C., L. N. Bald, B. M. Fendly, M. Mora-Worms, I. S. Figari, E. J. Patzer, and M. A. Palladino. The autocrine production of transforming growth factor-beta 1 during lymphocyte activation. A study with a monoclonal antibody-based ELISA. *J Immunol.* **145**:1415-22 (1990)

42. Snapper, C. M., W. Waegell, H. Beernink, and J. R. Dasch. Transforming growth factor-beta 1 is required for secretion of IgG of all subclasses by LPS-activated murine B cells in vitro. *J Immunol.* **151**:4625-36 (1993)

43. Lund, J., J. D. Pound, P. T. Jones, A. R. Duncan, T. Bentley, M. Goodall, B. A. Levine, R. Jefferis, and G. Winter. Multiple binding sites on the CH2 domain of IgG for mouse Fc gamma R11. *Mol Immunol.* **29**:53-9 (1992)

44. Ravetch, J. V., and J. P. Kinet. Fc receptors. *Annu Rev Immunol.* **9**:457-92 (1991)

45. Sandor, M., and R. G. Lynch. 1998. Fc gamma R on T cells, p. 169-183. *In* J. G. J. van de Winkel, and P. M. Hogarth (ed.), The immunoglobulin receptors and their physiological and pathological roles in immunity. Kluwer Academic Publishers, Dordrecht

46. Hora, K., J. A. Satriano, A. Santiago, T. Mori, E. R. Stanley, Z. Shan, and D. Schlondorff.. Receptors for IgG complexes activate synthesis of monocyte chemoattractant peptide 1 and colony-stimulating factor 1. *Proc Natl Acad Sci U S A.* **89**:1745-9 (1992)

47. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells.*Nat Immunol.* 1:311-6. (2000)

48. Nitta, T., and T. Suzuki.. Fc gamma 2b receptor-mediated prostaglandin synthesis by a murine macrophage cell line (P388D1). *J Immunol.* **128**:2527-32 (1982)

49. Unkeless JC, Eisen HN, Binding of monomeric immunoglobulins to Fc receptors of mouse macrophages. *J Exp Med.*142:1520-33 (1975)

50. Haeffner-Cavaillon N, Klein M, Dorrington KJ. Studies on the Fc gamma receptor of the murine macrophage-like cell line P388D1. I. The binding of homologous and heterologous immunoglobulin G1. *J Immunol* **123**:1905-13 (1979)

51. Kimura S, et al. A new mouse cell-surface antigen (Ly-m20) controlled by a gene linked to *Mls* Locus and defined by monoclonal antibodies. Immunogenetics 14: 3-14 (1981)

## Claims

1. A method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine comprising providing a ligand capable of modulating FcγRIIB receptor signalling.

2. A method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine according to claim 1, wherein said tolerance is modulated via mucosal administration of said ligand.

3. A method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine according to claim 1 or 2 further comprising said antigen.

4. A method for modulating tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder or an antigen used in a vaccine according to claim 3, wherein said tolerance is modulated via mucosal administration of said ligand and said antigen.

5. A method for inducing tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder comprising providing a ligand capable of inducing FcγRIIB receptor signalling and further comprising said antigen.

6. A method for inducing tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder according to claim 5, wherein said tolerance is induced via mucosal administration of said ligand and said antigen.

7. A method for inducing tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder according to claim 5 or 6, wherein said allergic reaction is asthma, eczema, hay fever, urticaria or food allergy.

8. A method for inducing tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder according to claim 5 or 6, wherein said

autoimmune disorder is rheumatoid arthritis, multiple sclerosis, Systemic lupus erythematosus, Sjögrens' syndrome, autoimmune diabetes, celiac disease or Crohn's disease.

9. A method for inducing tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder according to anyone of the claims 5 to 8, wherein said ligand comprises at least the Fc part of IgG2b and/or a functional derivative and/or a functional fragment thereof.

10. A method for preventing tolerance to an antigen used in a vaccine against an infection disease comprising providing a ligand capable of inhibiting FcγRIIB receptor signalling and further comprising said antigen.

11. A method for preventing tolerance to an antigen used in a vaccine against an infection disease according to claim 10, wherein said tolerance is prevented via mucosal administration of said ligand and said antigen.

12. A method for preventing tolerance to an antigen used in a vaccine against an infection disease according to claim 10 or 11, wherein said infection disease is influenza, Helicobacter pylori or pneumococcen.

13. A pharmaceutical composition comprising a ligand capable of inducing FcγRIIB receptor signalling and an antigen involved in an allergic reaction or in an autoimmune disorder.

14. A pharmaceutical composition according to claim 13, wherein said ligand comprises at least the Fc part of IgG2b and/or a functional derivative and/or a functional fragment thereof.

15. Use of a pharmaceutical composition according to claim 13 or 14 for the induction of (mucosal) tolerance to an antigen involved in an allergic reaction or in an autoimmune disorder.

16. Use of pharmaceutical composition according to claim 13 or 14 for reducing the effects of an allergic reaction or the effects of an autoimmune disorder.

17. Use according to claim 16, wherein said allergic reaction is asthma, eczema, hay fever, urticaria or food allergy.

18. Use according to claim 16, wherein said autoimmune disorder is rheumatoid arthritis, multiple sclerosis, Systemic lupus erythematosus, Sjögrens' syndrome, autoimmune diabetes, celiac disease or Crohn's disease.

19. A pharmaceutical composition comprising a ligand capable of inhibiting FcγRIIB receptor signalling and an antigen used in a vaccine against an infection disease.

20. A pharmaceutical composition according to claim 20, wherein said ligand comprises an antibody against the FcγRIIb receptor and/or a functional derivative and/or a functional fragment thereof.

21. Use of a pharmaceutical composition according to claim 19 or 20 for preventing tolerance to an antigen used in a vaccine against an infection disease.

22. Use according to claim 21, wherein said wherein said infection disease is influenza, Helicobacter pylori or pneumococcen.

23. A method for identifying a ligand of FcγRIIB receptor signalling by screening a compound library comprising

- culturing cells comprising a FcγRIIB receptor
- providing a testcompound to said cells
- testing medium surrounding said cells for cytokine and/or chemokine levels.

24. A ligand obtainable by a method according to claim 23.

FIGURE 1

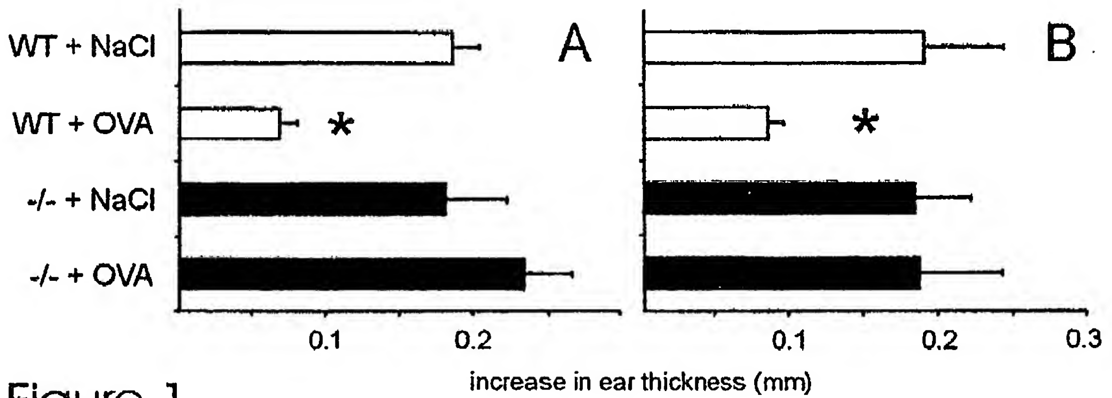



Figure 1

**FIGURE 2**

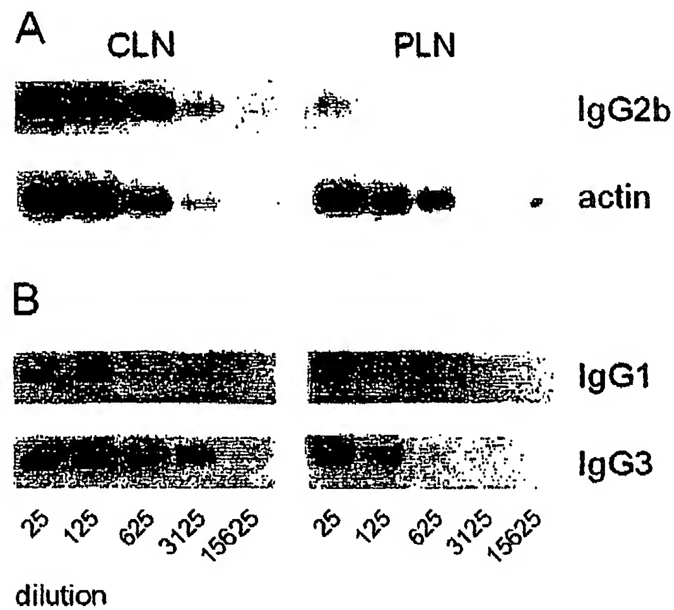



Figure 2

# FIGURE 3

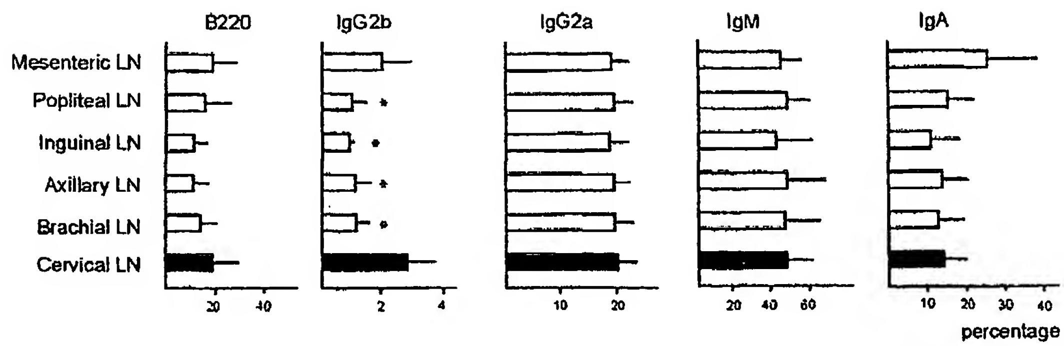



Figure 3

FIGURE 4

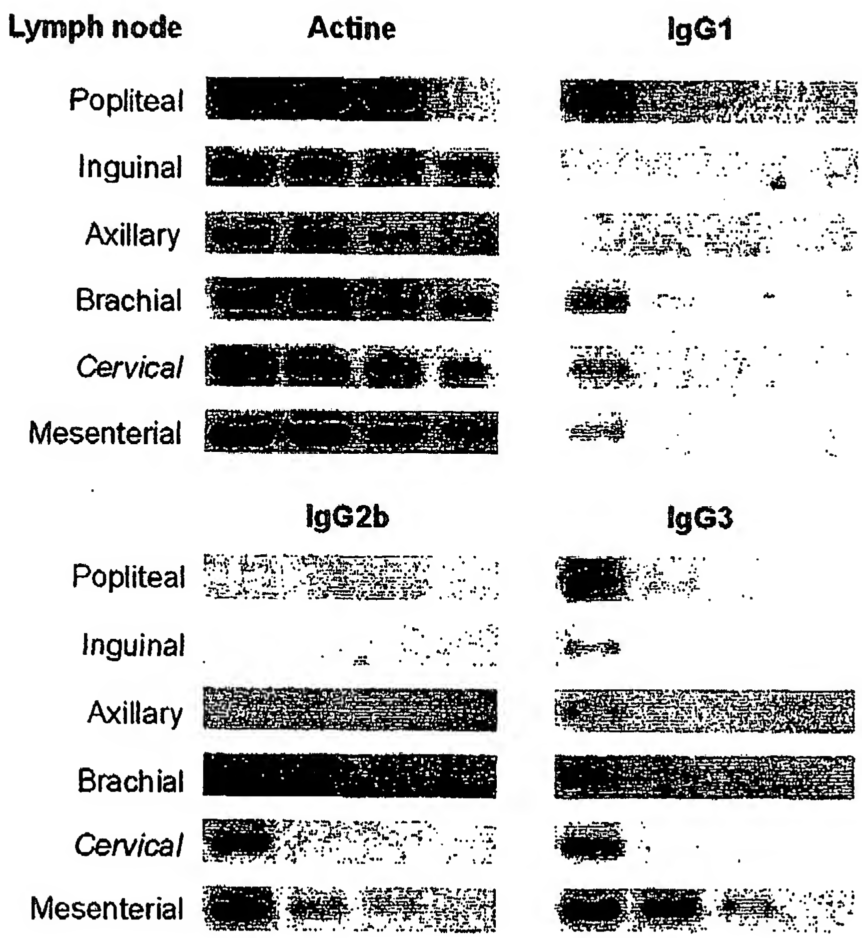



Figure 4

**FIGURE 5**

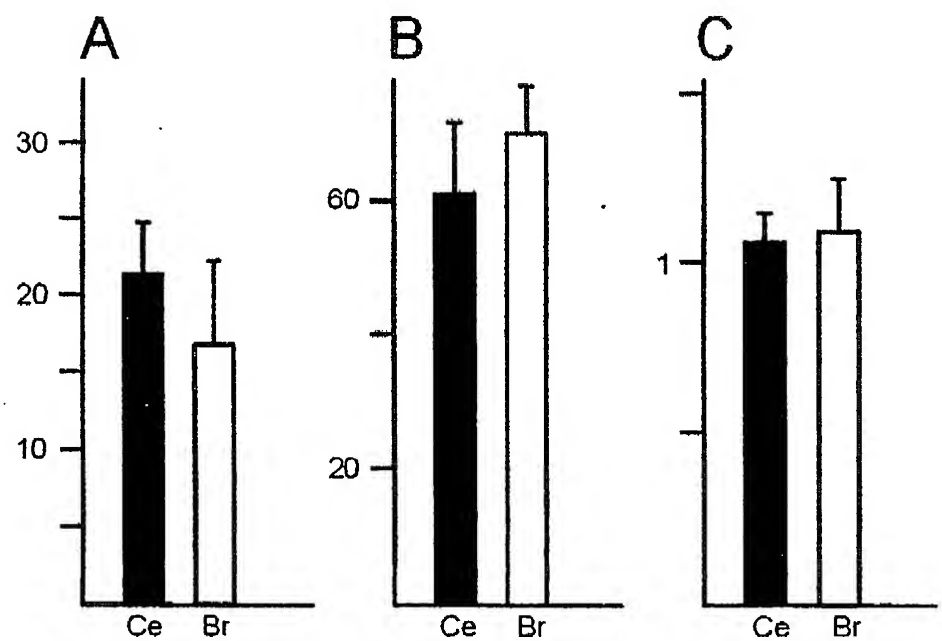



Figure 5

FIGURE 6

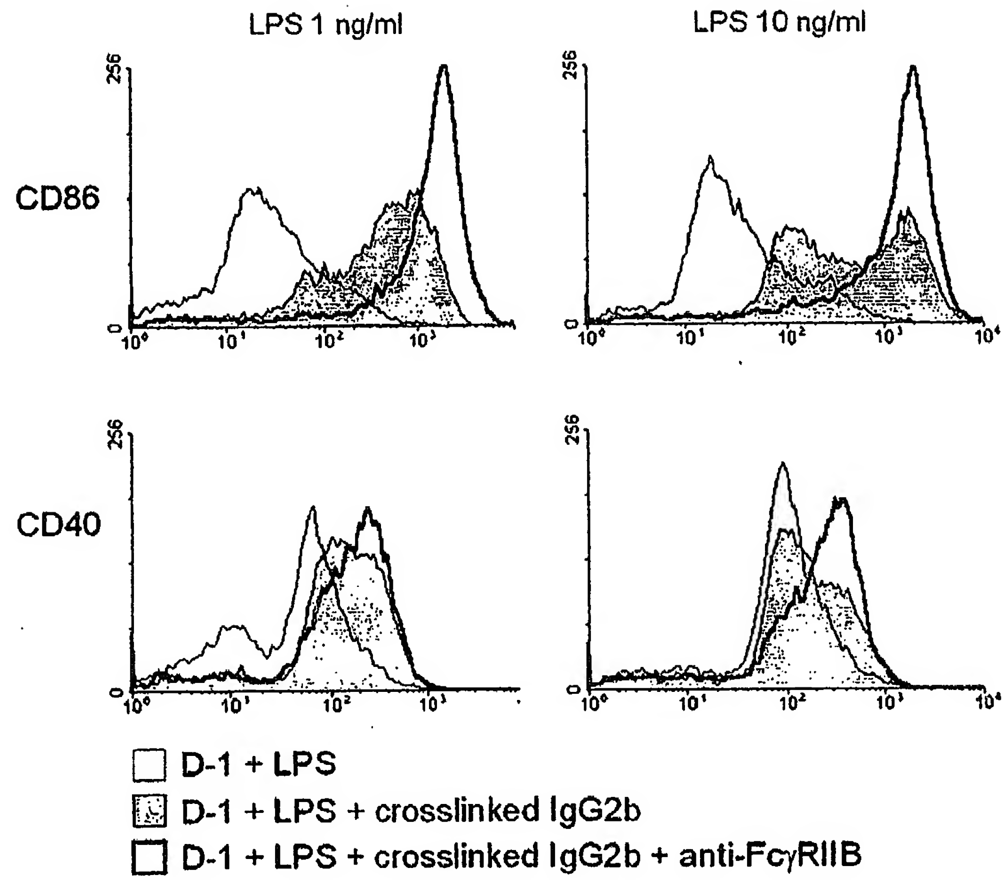
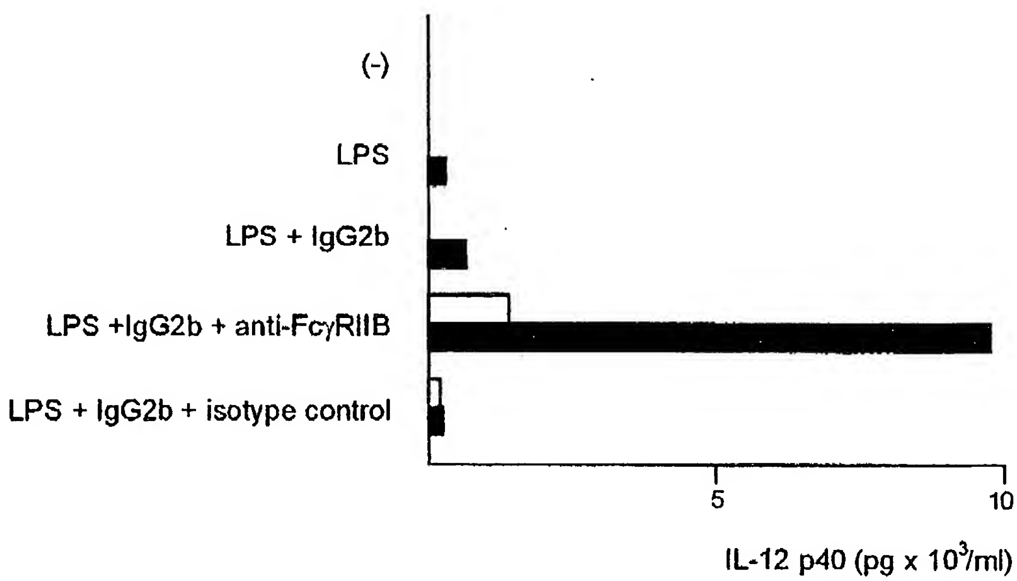



Figure 6

FIGURE 7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